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(54) Title: MINERALIZED COLLAGEN-POLYSACCHARIDE MATRIX FOR BONE AND CARTILAGE REPAIR

(57) Abstract: A matrix and a method for preparing it are provided to support the growth of tissue, such as bone, cartilage or soft connective tissue. A polysaccharide is reacted with an oxidizing agent to open sugar rings on the polysaccharide to form aldehyde groups. The aldehyde groups are reacted to form covalent linkages to mineralized collagen. The matrix can be implanted or injected, or the polyaldehyde polysaccharide and mineralized collagen starting minerals can be separately injected to form the matrix *in situ*.

MINERALIZED COLLAGEN-POLYSACCHARIDE MATRIX FOR BONE AND CARTILAGE REPAIR

FIELD OF THE INVENTION

The present invention is directed to crosslinked mineralized collagen-polysaccharide matrices for the therapeutic repair of tissue, such as, bone, cartilage and soft tissue; methods of producing such matrices; and methods of using the matrices to repair tissue. The present invention provides a crosslinked mineralized collagen-polysaccharide matrix that is administered by implantation or injection alone or in combination with other therapeutics, such as growth factors, for tissue repair.

BACKGROUND OF THE INVENTION

There is a clinical demand for a bone grafting matrix that offers osteoconductive properties equal to autogenous bone and that can be produced in unlimited supply. Although some bone substitutes are available, many consist of materials that have poor physical handling and resorption characteristics that complicate their use and radiographic evaluation.

Similarly, there is no consistently effective commercial product that supports the differentiation or maintenance of the chondrocyte phenotype of cartilage tissue, despite years of extensive research. Prior strategies to facilitate the repair of damaged cartilage have included the transplantation of existing host cartilage and/or the implantation of prosthetic devices. Limitations of these methods are the availability of donor tissue and the limited lifespan of prosthetic implants. More recently, the *ex vivo* cultivation of mature chondrocytes on polymeric scaffolds has been used in an attempt to generate cartilage graft material but this has not yet been widely accepted in part because it involves two surgical procedures: one to harvest chondrocytes and the second to implant them after expansion *in vitro*.

Collagens and glycosaminoglycans are two classes of biomaterials suited for use in bone and cartilage regeneration. Collagen based matrices have been used in bone grafting. Type I collagen has good cell adhesive properties, in particular, for bone forming osteoblast cells. Collagen has the capacity to serve both as an active or inert scaffold material for growth.

Bone is characteristically composed of type I collagen fibrils intimately associated in an orderly manner with calcium phosphate crystals. Minor constituents include an array of macromolecules as well as a series of small molecules associated mainly with the mineral phase.

One feature of bone is the exceedingly small size of the crystals. Bone crystals are among the smallest biologically formed crystals known and, in fact, crystallographers would intuitively not expect crystals just a few unit cells thick to be stable at all. Therefore, the collagen bone structure has unique characteristics as to its formation, components, and properties.

Mineralized collagen having calcium phosphate stably dispersed in an ordered manner associated with collagen fibrils is disclosed in U.S. 5,231,169, which is incorporated-by-reference herein in its entirety.

Hyaluronic acid is a natural component of the cartilage extracellular matrix, and it is readily sterilized, is biodegradable and can be produced in a wide range of consistencies and formats. It is generally biocompatable and its resorption characteristics can be controlled by the manipulation of monomers to polymer forms, most commonly through the esterification of the carboxylic groups of the glucuronic acid residues.

Biological glue comprising fibrin has a long history as a tissue adhesive medical device and is believed to be commercially available in Europe (United States patent No. 5,260,420, issued November 9, 1993). One obstacle that limits its application is the short turn over and residence time which ranges from a few days to a few weeks depending on the site of implantation. The incorporation of collagen fibers into fibrin glue has been reported (Sierra et al., 1993, Trans. Soc. Biomater., vol. 16:257 and United States Patent No. 5,290,552). However, longer coagulation times are required for the collagen/fibrin compositions compared to fibrin alone.

There remains a need for biodegradable, biocompatable matrices which maintain structural integrity and which can be used to repair tissues without resorting to undesirable *ex vivo* cultivation methods.

SUMMARY OF THE INVENTION

The present invention provides crosslinked mineralized collagen-polysaccharide matrices, methods for preparing such matrices, and methods of using the matrices by implantation or injection in the repair of tissue, such as, bone, cartilage and other soft connective tissue. The mineralized collagen may be formed from purified, native, modified or recombinant collagen of any type. The type of polysaccharides which can be used include hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan, heparan sulfate, dextran, dextran sulfate, alginate, and other long chain polysaccharides. In a preferred embodiment, the polysaccharide is hyaluronic acid.

A crosslinked mineralized collagen-polysaccharide matrix of the present invention may be used alone to conduct the growth of tissue; or in combination with growth factor.

Growth factors which can be used with a matrix of the present invention include, but are not limited to, members of the TGF-ß superfamily, including TGF-ß1,2 and 3, the bone morphogenetic proteins (BMP's), the growth differentiation factors(GDF's), and ADMP-1; members of the fibroblast growth factor family, including acidic and basic fibroblast growth factor (FGF-1 and -2); members of the hedgehog family of proteins, including indian, sonic and desert hedgehog; members of the insulin-like growth factor (IGF) family, including IGF-I and -II; members of the platelet-derived growth factor (PDGF) family, including PDGF-AB, PDGF-BB and PDGF-AA; members of the interleukin (IL) family, including IL-1 thru -6; and members of the colony-stimulating factor (CSF) family, including CSF-1, G-CSF, and GM-CSF.

The method of making a mineralized collagen-polysaccharide matrix of the present invention comprises the steps of oxidizing an exogenous polysaccharide to form a modified exogenous polysaccharide having aldehyde groups, and reacting the modified exogenous polysaccharide with mineralized collagen under conditions such that the aldehyde groups covalently react with mineralized collagen to form a crosslinked matrix. The method may further comprise the step of adding a growth factor to the matrix. A growth factor can be added before or after the step of reacting the modified polysaccharide with the mineralized collagen. The mineralized collagen is prepared from dispersed or solubilized collagen according to the method of U.S. Patent 5,231,169.

The present invention provides methods of using a crosslinked mineralized collagen-polysaccharide matrix to conduct the growth of tissue by administering the matrix at the sites of desired tissue repair. The matrix in combination with a growth factor may be administered by implantation or injection to induce the growth of tissue at sites of desired repair. A matrix further comprising fibrin may be administered to anchor the matrix into desired sites, such as, tissue defect sites.

The prepared matrices may be implanted at a site of desired tissue growth. The polysaccharide and mineralized collagen starting materials may also be separately injected into the site of desired tissue growth, along with any growth factor, and the like. Upin mixing at the site, the matrix will form *in situ*, conforming to the shape of the site.

As used in this discussion, "repair" is defined as growth of new tissue. The new tissue may or may not be phenotypically or genotypically identical to the original lost tissue. As used herein, "regeneration of tissue" means that the new tissue grown is identical to the lost tissue. Tissue repair can also be the result of replacing lost tissue with non-identical tissues, e.g., for example, the replacement of hyaline articular cartilage with fibrocartilage in a joint defect. The basic cellular properties involved in repair include adhesion, proliferation, migration and differentiation.

By "conduction", it is meant that the host tissue, e.g.,bone, cartilage or soft tissue grows by extension of existing tissue onto or into the crosslinked collagen-polysaccharide matrix. In conduction, repair cells move onto and into the matrix to synthesize and remodel new tissue identical to the surrounding host tissue. By induction, it is meant that the growth and differentiation of progenitor repair cells is stimulated. These progenitor cells go on to synthesize and remodel new tissue to be continuous with the surrounding host tissue.

As used herein, a tissue defect can be the result of a congenital condition, trauma, surgery, cancer or other disease.

As used in this discussion, an exogenous polysaccharide refers to a free polysaccharide.

The ratios of the mineralized collagen to polysaccharide can be varied to change both the physical and biological properties of the matrix. A higher proportion of mineralized collagen will result in a more porous sponge-like matrix. A higher proportion of polysaccharide will result in a more gel-like matrix.

BRIEF DESCRIPTION OF THE DRAWING

The Figure is a graph of the comparative release rates of GDF-5 from three matrices as described in Example 9.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The method of preparing a matrix of the present invention comprises the steps of opening sugar rings on an exogenous polysaccharide and oxidizing terminal hydroxyl groups to aldehydes using, for example, sodium or potassium periodate as a selective oxidizing agent. The amount of aldehyde groups produced in this manner can be stoichiometrically controlled. Typically, from about 1% to 50% of the rings can be opened in this manner. More preferably about 1% to 5% of the rings are opened to form the aldehyde groups. These aldehyde groups can form covalent crosslinks with the collagen at amine sites on the collagen peptide chains. Since the aldehyde groups are formed *in situ* without the addition of a separate cross-linking compound, the intermolecular distance between the backbone of the polysaccharide chain and the collagen fibrils which is crosslinked to it is believed to be less than the corresponding distance using a crosslinking compound. Accordingly, the polysaccharide and collagen backbones are relatively closely bound, which produces an advantageous structure for the purpose of providing a matrix that supports, conducts or induces the growth of bone, cartilage or soft connective tissue.

The starting material for producing the collagen may be purified, native collagen, modified or recombinant collagen of any type. A preferred collagen for bone growth is Type I collagen, whereas a preferred collagen for cartilage growth is Type II collagen. The collagen may be crosslinked or non-cross-linked, but it is preferred that the collagen be non-crosslinked to provide more accessibility to side groups for crosslinking to the polysaccharide aldehyde groups. If Type I collagen is used for tissue repair where it is desired to mask the inherent cell adhesion sites, such as cartilage repair, the adhesion sites can be masked by the use of non cell-adhesive polysaccharides to support the increased cell-to-cell interaction and adhesion.

The collagen to be mineralized will normally be dispersed or solubilized collagen where solubilization is achieved by dispersing the collagen source in a medium at an elevated pH, using at least about pH 8, more usually about pH 11-12, and generally less than about 1 N. Commonly, sodium hydroxide is employed, although other hydroxides may find use, such as other alkali metal hydroxides or ammonium hydroxide.

The concentration of collagen will generally be in the range of about 1 to 10 weight percent, more usually from about 1 to 5 weight percent. The collagen medium will generally be at a concentration of the base in the range of about 0.0001 to 0.1 N. The pH is generally maintained during the course of the reaction in the range of about 10-13, preferably about 12.

The phosphate and calcium are added as solutions, generally at a concentration in the range of about 0. 010.2 M, preferably about 0.025-0.075 M. The volume of the solutions added to the collagen medium will generally increase the collagen medium volume by at least 10 percent, usually at least 25 percent and not more than about 400 percent, generally in the range of about 50 to 150 percent. Thus, the collagen solution will generally not be diluted by more than four-fold.

The rate of addition is relatively slow, generally requiring at least about one hour and not more than about 72 hours, generally being in the range of about 2 to 18 hours, more usually in the range of about 4 to 16 hours. For example, with one liter of a collagen dispersion, where about a total of about one liter of reagents is added, the rate of addition will generally be in the range of 50 to 150 ml per hour.

The addition of the reagents can be provided in a stoichiometric ratio, although stoichiometry is not required, variations from stoichiometry of up to about 50 percent, preferably not more than about 25 percent are preferred. Thus, where the stoichiometry of addition is not maintained, one of the components may be exhausted, while addition of the other components continue.

During the course of the reaction, mild agitation is maintained, so as to ensure substantially uniform mixing of the collagen fibrils and the calcium phosphate mineral. Mild temperatures are employed, usually not less than about 4°C and not more than about 40°C preferably in the range of about 15°C to 30°C. The weight ratio of the collagen to calcium phosphate mineral will generally be in the range of about 8:2 to 1:1, more usually about 7:3.

After completion of the addition, agitation, e.g., stirring, will normally be continued, usually at least about 1 h, more usually about 2 h and agitation may continue even more. The amount of continued agitation is not critical to the preparation of the product.

Upon completion of the reaction, the mineralized collagen may be treated in a variety of ways. The product may be washed repeatedly to remove any unbound minerals or other components of the medium, as well as provide a more neutral pH. Washing may be readily accomplished with water, saline, or the like.

The mineralized collagen may be further treated in a variety of ways. The subject compositions may be cross-linked using a variety of cross-linking agents, such as formaldehyde, glutaraldehyde, chromium salts, di-isocyanates or the like.

It is a feature of the invention that the polyaldehyde polysaccharide and mineralized collagen are separately injectable materials which can react when contacted to form the matrix *in situ* at the site of desired tissue growth. The advantages are that no operative implantation procedures are necessary and the flowable starting materials conform to the shape of the site before the reaction is complete. The result is a matrix that conforms in shape to the site without the need for cutting and shaping of a pre-formed solid matrix.

The type of polysaccharides which may be utilized include hyaluronic acid, chondroitin sulfate, dermatan, dextran sulfate, alginate, and other long chain polysaccharides. Typically, the polysaccharide will have an average molecular weight of about 1,000 to 10,000,000 DA.

The reagents for opening sugar rings on the exogenous polysaccharide may be any selective oxidizing agent which oxidizes a terminal hydroxyl group to an aldehyde, such as potassium or sodium periodate. Other reagents include specific sugar oxidases.

The preferred polysaccharide is hyaluronic acid. The relative proportion of polysaccharide to mineralized collagen will impart various physical and biological characteristics to the matrix. The proportion of polysaccharide to mineralized collagen may be characterized on a molar ratio basis or on a weight ratio basis. Typically, the ratio by weight of mineralized collagen to polysaccharide is from 99:1 to about 1:99. This represents an approximate molar ratio of 99.9:0.1 to 1:9, respectively, assuming an average molecular weight of 1,000,000 daltons for hyaluronic acid and 100,000 daltons for the collagen (based on non-mineralized weight). The molar ratio may vary depending on the actual molecular weight of the polysaccharide and collagen used. In a preferred embodiment disclosed herein, the ratio by weight of collagen to polysaccharide is from 9:1 to about 1:9.

The ratios of the mineralized collagen to polysaccharide can be varied to change both the physical and biological properties of the matrix. Biologically, a higher proportion of Type I collagen will more closely mimic the composition and architecture of bone, whereas a higher proportion of Type II collagen will more closely mimic the composition of cartilage. Bone forming cells will interact with specific cell adhesion sites on collagen and will divide, migrate and differentiate to form new bone.

Alternatively, increasing the proportion of polysaccharide, preferably hyaluronic acid, will more closely mimic a natural cartilage matrix. In addition, a higher proportion of polysaccharide will mask some specific cell adhesive sites on collagen and will favor other cell-cell interactions and aggregation important in the development of cartilage tissue.

Growth factors which can be used with a matrix of the present invention include, but are not limited to, members of the TGF-ß superfamily, including TGF-ß1,2 and 3, the bone morphogenetic proteins (BMP's), the growth differentiation factors(GDF's), and ADMP-1; members of the fibroblast growth factor family, including acidic and basic fibroblast growth factor (FGF-1 and -2); members of the hedgehog family of proteins, including indian, sonic and desert hedgehog; members of the insulin- like growth factor (IGF) family, including IGF-I and -II; members of the platelet-derived growth factor (PDGF) family, including PDGF-AB, PDGF-BB and PDGF-AA; members of the interleukin (IL) family, including IL-1 thru -6; and members of the colony-stimulating factor (CSF) family, including CSF-1, G-CSF, and GM-CSF. Growth factor preparations are obtained either commercially or isolated and purified from tissue or from recombinant sources. Growth factors can be loaded into the collagen/HA/fibrin matrices across a wide dose range (fentogram to millgram range). Factors such as cost, safety and the desired growth factor release profile will dictate the amount of growth factor that is loaded onto the matrix.

Thrombin acts as a catalyst for fibrinogen to provide fibrin. In the present invention, the fibrinogen and thrombin are added individually to a reaction mixture containing oxidized exogenous polysaccharide and mineralized collagen. In this embodiment, it is desired to keep the fibrinogen and thrombin separated and the oxidized, exogenous polysaccharide and mineralized collagen separated until final reaction is desired.

The concentration of fibrinogen used in forming the matrix is preferably 10 mg/ml or greater. The thrombin is added to the fibrinogen in a concentration of from about 0.01 NIH units to about 100 NIH units/ml and preferably from about 0.1 - 2.0 NIH units/ml. The thrombin is commercially available from a variety of sources. Fibrinogen may be derived from autologous patient plasma or from commercial sources. The matrices according to the present invention may be formed into any shape by lyophilization, or wet-laying and air drying in molds of the desired shape. The wet-laid material having a high proportion polysaccharides may also be formed into viscous gels for injection or direct application into a fracture or joint. As described above, the starting materials are also injectable and may be separately injected to mix at the site of desired tissue growth to react without formation of undesired side products.

The usefulness of the matrices according to the present invention can be shown by both *in vitro* and *in vivo* tests. For the *in vitro* tests, primary fetal rat calvarial cells, harvested by a series of collagenase digestions, according to the method of Wong and Cohn (PNAS USA 72:3167-3171, 1975), or primary rat epiphyseal cartilage Thyberg and

Moskalewski, (Cell Tissue Res. 204:77-94, 1979) or rabbit articular chondrocytes, harvested by the method of Blein-Sella O. et al., (Methods Mol. Biol., 43:169-175, 1995), are seeded into the matrices and cultured under conventional conditions for 1-4 weeks. Cultures are then processed and evaluated histologically.

A particular advantage of the polysaccharide mineralized collagen matrices according to the invention is that they have comparable growth factor binding ability to cross-linked mineralized collagen but much better osteoconductivity. Furthermore, while the polysaccharide-mineralized collagen matrices have comparable osteoconductivity to polysaccharide-nonmineralized collagen, they have slower growth factor release kinetics, which is an advantage for growth factor retention within the matrix.

The chondroconductive capability of the matrices of the present invention can be determined by successful support of adhesion, migration, proliferation and differentiation of primary rat bone marrow and stromal cells as well as retinoic acid-treated primary rat or rabbit chondrocytes or human mesenchyme stem cells. Bone marrow and bone marrow stromal cells closely approximate the early chondroprogenitor cells found in the subchondral bone marrow of full-thickness defects. Bone marrow are harvested from the long bones of 2-3 week-old inbred Lewis rats and added directly to a matrix and cultured for 2 weeks under standard conditions. The adherent stromal cell population that grows out of these cultures are passaged and frozen for use. Cells from up to six passages are used for culturing or seeding on the matrix.

Retinoic acid-treated chondrocytes represent the latter stages of chondrogenesis. Retinoic acid treatment of primary is performed prior to culturing or seeding the cells on a candidate matrix (Dietz, U. et al., 1993, J. Cell Biol. 52(1):57-68).

In an alternative method, *in vitro* studies of the early and late stage chondrocytes are merged to allow stromal cells to condition the matrices and then to replace them with more mature chondrocytes. In this way, evolution of the matrices during the early phases of chondrogenesis may be tested for effects on the later stages of the process.

Cell adhesion and proliferation on the matrix are monitored using an MTS assay that can measure cell number and viability based on mitochondrial activity. Stromal cells or chondrocytes are cultured on matrices for 6-18 hrs. in the presence or absence of serum for adhesion analysis and for 1-2 weeks for proliferation assessment.

For cell migration testing, matrices are coated or fitted onto porous Trans-well membrane culture inserts (Corning). Stromal cells are seeded on top of the matrices in the upper chamber of the Trans-well and a chemoattractant (growth factor, PDGF) placed in the bottom chamber. After 12-18 hrs of culture the cells that have migrated through the matrix to the bottom side of the Trans-well membrane are quantitated by the MTS assay. Matrices are removed from the upper chamber and processed histologically to assess degree of infiltration.

The analysis of differentiation markers relevant to chondrogenesis and osteogenesis are evaluated at both the protein and transcriptional level. The specific markers that may be analyzed include: 1) Type II collagen and IIA, IIB isoforms; 2) Aggrecan proteoglycan; 3) Type IX, X and XI collagen; 4) Type I collagen; 5) Cartilage matrix protein (CMP); 6) Cart-1 transcription factor; 7) Fibronectin (EDA, EDB isoforms); 8) Decorin proteoglycan; 9) Link protein; 10) NG-2 proteoglycan; 11) Biglycan proteoglycan; 12) Alkaline phosphatase. Differentiation may be measured by Northern/PCR analysis, Western blotting or by metabolic cell labeling.

For Northern/PCR analysis, RNA are isolated by standard procedures from stromal cells or chondrocytes that have been cultured on composite matrices. Time course tests may be used to determine optimal culture periods that range from 1 to 6 weeks depending on the cell type. The isolated RNA is analyzed by Northern gel and hybridization techniques with specific cDNA or PCR amplified probes. Northern analysis is quantified by densitometric scanning of autoradiographs and normalization to housekeeping gene signals (G3PDH). Northern analysis may be supplemented with quantitative PCR analysis using primers generated from the published cDNA sequences of the genes to be analyzed.

For Western blotting, solubilized protein lysates are isolated from cells cultured on composite matrices by standard techniques (Spiro R.C., et al., 1991, J. Cell. Biol., 115:1463-1473). After the lysis of cells the matrices are extracted in stronger denaturants (8 M urea, GnHCL) to remove and examine matrix-bound or incorporated proteins. Protein samples are analyzed by standard Western blotting techniques using specific polyclonal or monoclonal antibodies.

For metabolic cell labeling, cells cultured on a composite matrix are metabolically radiolabeled with $^{35}SO_4$, ^{35}S-methionine or ^{3}H/^{14}C-labeled amino acids by standard techniques (Spiro et al., supra). Solubilized cellular and matrix-associated proteins are quantitatively immunoprecipitated with antibodies specific for the protein of interest and analyzed by SDS-PAGE (Spiro et al., supra). Quantitation of results are performed by densitometric scanning of autoradiographs and signals will be normalized to either cell equivalents or to a house-keeping protein such as actin.

Additionally, the ability of a matrix of the present invention to support chondrogeneic differentiation *in vivo* may be tested in an inbred rat soft tissue implant model. Rat bone marrow or stromal cells described above are seeded onto matrices at high density, cultured overnight in MEM medium containing 10% FBS serum and antibiotics, then transferred into Millipore diffusion chambers and implanted intraperitoneally or subcutaneously into 8 week-old recipients. Chambers are harvested after 3 weeks and evaluated histologically for cartilage formation.

A transplantation model in outbred rats is used to evaluate the ability of the composite matrices to maintain the cartilage phenotype *in vivo*. Rib costal cartilage chondrocytes are seeded onto matrices at high density and cultured overnight in Ham's F-12 containing 1% rat serum and antibiotics. The seeded matrices are then implanted into posterior tibial muscle pouches created by blunt dissection in 8 week-old male Sprague-

Dawley rats. Explants are taken at 14 and 28 days and evaluated histologically for matrix compatibility, cartilage growth, and maintenance of the differentiated phenotype based on staining for aggrecan and type II collagen.

In addition, the ability of a matrix of the present invention to interact with extracellular matrix proteins (proteoglycans, proteins and growth factors) found in the surrounding serum, tissue fluid, or in the secretion products of chondroprogenitor cells correlate with the chondroconductive potential of a matrix. The interaction of the matrices of the present invention with extracellular matrix proteins may be measured by means known to those of skill in the art such as, Western blotting, affinity co-electrophoresis techniques and binding characteristics.

To assay serum protein binding to a matrix of the present invention, the matrix is incubated in culture media containing increasing amounts of serum (various species and sources). After washing, bound proteins are eluted by boiling in SDS-PAGE sample buffer and unsolubilized matrix will be removed by centrifugation. SDS-PAGE analysis is used to initially document the binding pattern of the matrices. Western blotting is then performed to identify specifically bound components such as fibronectin and vitronectin.

Affinity coelectrophoresis is used to analyze proteoglycan binding to a matrix of the present invention. $^{35}SO_4$-labeled or iodinated proteoglycan (aggrecan) isolated from bovine and rat (or other sources) is loaded into ACE gels (Lee, M.K. et al., 1991, 88:2768-2772) containing composite matrices or collagen scaffolds alone. The binding affinity of aggrecan for collagen scaffolds plus and minus hyaluronic acid or dextran sulfate are taken as a measure of the ability of composite matrices to organize a cartilage matrix.

An evaluation of protein interactions with mineralized collagen-based composite matrices can potentially be hindered by the large excess of collagen protein. The mineralized collagen scaffolds have enough inherent structural integrity and are crosslinked to an extent that will prevent their complete solubilization, but some collagen protein may become solubilized in the SDS-PAGE sample buffer. Thus, this could obscure the visualization of other bound proteins, particularly the cell-synthesized collagens, and may also cause high background in Western blot analysis. Therefore, an alternative approach is to use radiolabeled or biotinylated proteins for the binding analysis. Serum proteins may be biotinylated prior to incubation with the composite matrices and then developed with avidin-based reagents. Both approaches allow the visualization of matrix-associated components without the interference of the scaffold collagen protein.

Alternatively, the shift in expression from Type I to Type II collagen and the splicing of the Type II collagen transcript from the Type IIA to the Type IIB isoform (Sandell, L.J. et al., 1991, J. Cell Biol. 114:1307-1319) are measured by means known to those of skill in the art to determine differentiation down a chondrogenic pathway. Also, the expression of the cartilage-associated proteoglycan, aggrecan (Schmid, T.M., et al., 1985, J. Cell Biol. 100:598-605 and Kuettner K.E. 1992, Clin. Biochem. 25:155-163) and a cartilage homeoprotein transcription factor (Cart-1) appear to be markers for cells committed to the chrondrocytic lineage.

For the *in vivo* tests, the matrices are evaluated for the capabilities for supporting osseous healing in a rat cranial defect model by implantation into a 5 mm by 3 mm defect created in the parietal bone of 6 weeks old male Sprague-Dawley rats. The defects are evaluated at 28 days by radiographic and histologic analysis.

The *in vivo* model for cartilage repair is a full-thickness articular cartilage defect in the rabbit (Amiel et al., 1985, J. Bone Joint Surg. 67A:911). Defects measuring approximately 3.7 mm in diameter and 5 mm deep defect are created in the center of the medial femoral condyles of adult male New Zealand white rabbits. The defects are then either filled with matrix or left unfilled as controls. The defects are evaluated morphologically and histologically at 6 and 12 weeks.

The matrices of the present invention may be used for the treatment of bone and/or cartilage defects associated with surgical resection, such as spinal fusions; trauma; disease; infection; cancer or genetic defects. The matrices according to the present invention may be administered through implantation, direct application or injection depending on the intended application of the matrix, the physical properties of the matrix and the ratio by weight of mineralized collagen to polysaccharide in the matrix.

In one aspect of the present invention, the matrix is provided having a higher proportion of mineralized collagen compared to polysaccharide, is in a sponge-like form and is surgically implanted at a site where growth of new bone tissue is desired, such as in spinal fusions. In one aspect, the matrix further comprises a growth factor, such as BMP-2. In another aspect, the matrix further comprises fibrin to facilitate anchoring of the matrix into the desired site. In another aspect of the invention the starting polyaldehyde polysaccharide and mineralized collagen are separately injected into the site of the desired tissue growth along with any desired growth factors. The materials react *in situ* to form the matrix at the desired site.

In another aspect of the present invention, the matrix has a higher proportion of polysaccharide compared to mineralized collagen, is formed into a viscous gel and is either directly applied or injected into a site where growth of new bone tissue is desired, such as in filling bone defects, fracture repair and grafting periodontal defects. In yet another aspect of the present invention, the matrix is provided with a higher proportion of polysaccharide, is formed into a viscous gel and is injected directly or delivered through an arthoscopic procedure into a site where growth of cartilage tissue is desired, such as in injury induced cartilage damage or disease-induced cartilage damage such as in, osteoarthritis or rheumatoid arthritis.

As will be understood by those of skill in the art, the amount of matrix to be administered to conduct growth of bone or cartilage tissue depends upon the extent of the bone or cartilage defect to be treated. As will also be understood by those of skill in the art, the cost, safety, and desired growth factor release profile will dictate the type and amount of growth factor that is loaded onto the matrix.

The following examples are provided for purposes of illustration and are not intended to limit the invention in any way.

Example 1

Implantable Matrices of Amine-linked Mineralized Collagen and Polysaccharides

The raw materials, mineralized Type I collagen and polysaccharide-polyaldehyde, were prepared by the methods disclosed in U.S. Patent No. 5,231,169 and U.S. Patent No. 5,866,165, respectively. Mineralized semed F collagen (63 mg/ml) was mixed with a hyaluronate-polyaldehayde solution (7 mg/ml, 5% repeating units were oxidized; pH 7.5-9.0) at the equal volume ratio in the container of a heavy duty blender. Sodium cyanoborohydride ($NaCNBH_3$ 5.0 M in 1.0 M NaOH) was added to the mixture to the final concentration of 10 mM. The mixture was then blended 3 times at low speed for 10 seconds. The reaction was continued carrying on by pouring the slurry into a heavy-wall bottle incorporated with a tight-fitting polypropylene screw cap. The bottle was rotated at the speed of 100 rotes/min. at ambient temperature in dark for 24 hr. The slurry was then poured into a mold and lyophilized. This formed a matrix, which was washed with D.I. water to removed $NaCNBH_3$ and re-lyophilized. This procedure was followed to make a series of matrices from mCOL with other oxidized polysaccharides. The surface property, structures and biological activity of the matrices were controlled by altering the ratio of mCOL to the polysaccharides, the type of polysaccharides, the density of aldehyde groups generated on the polysaccharides, the density of matrix, as well as the process of lyophilization.

Example 2

Implantable Matrices of Imine-linked Mineralized Collagen and Polysaccharides

The matrices were prepared by the procedure as described in Example 1, except that no $NaCNBH_3$ was used.

Example 3

Injectable Gel-Matrices with Fibrin

The gel-matrices were prepared using a FibrinJet™ surgical sealant delivery system. The typical procedure in detail is described in following:

The mCOL fibers with the diameter of about 100 μm and fibrinogen (mCOL, 42 mg/ml; fibrinogen, 21 mg/ml; pH 7.5) were loaded in one syringe, an equal volume of activated hyaluronate solution (7 mg/ml, pH 7.5) containing thrombin (1-5 U/ml) was placed in another syringe. Both of the syringes were mounted onto the FibrinJet™ surgical sealant delivery system connected with a 18 G needle. Gel-matrix was formed in less than three minutes at the exit site of the needle upon pushing the two parts of composition to flow through the syringes simultaneously.

The above procedure was followed to make a series of injectable gel-matrices from mCOL, fibrinogen, and other oxidized polysaccharides. The surface property, the porous structures and biological activity of the gel-matrices were controlled by altering the ratio of mCOL and polysaccharides to fibrinogen, the type of polysaccharides, the density of aldehyde groups, the density of the final matrix and the concentration of thrombin. The gelation time and the gel hardness were controlled by amount of thrombin added.

Example 4

Injectable Gel-Matrices with Plasma

The gel-matrices were prepared by the method described in Example 3, except that blood plasma was used instead of fibrinogen. The blood plasma was prepared from citrate (10 w%) added whole blood, which was centrifuged at 3,000 rpm for 15 minutes.

Example 5

Injectable Gel-Matrices with Plasma

The gel-matrices were prepared by the method described in Example 4, except that 50 mM calcium chloride solution was used instead of thrombin.

Example 6

Injectable Gel-Matrices with Whole Blood

The gel-matrices were prepared by the method described in Example 3, except that whole blood was used instead of blood plasma.

Example 7

Implantable Matrices with Growth Factors

The typical procedure to load growth factors onto the matrices is described as follows:

Matrices prepared from mCOL and active polysaccharides as prepared in Examples 1 and 2 were used. Pre-dried matrices were cut to cubes with the size of 5 x 4 x 2 mm. The water uptake of the cubes was measured and found to be 85 ± 5 μ per piece, Growth and differentiation factor-5 (GDF-5, 0.588 mg/ml, 20 mM acetic acid) was drop-wise added to the matrix specimens at 85 μl for each piece. The GDF-5 loaded matrix specimens were allowed to stand at ambient temperature in hood for 5 minutes, then froze at -78°C, and lyophilized.

The above procedure was followed to load a series of growth factors with various concentrations, such as bone morphogenic proteins, transferin growth factor-β, and insulin-like growth factors to matrices prepared from mCOL with other oxidized polysaccharides.

The above procedure was followed to load DNA, hormones, and cytokines to matrices prepared from mCOL with other oxidized polysaccharides.

Example 8

Injectable Gel-Matrices with Growth Factors

The typical procedure to load growth factors into injectable gel-matrices is described as follows:

mCOL fibers (D.I. < 100 μm, 42 mg/ml), fibrinogen (21 mg/ml), and GDF-5 (100 μg/ml) were loaded in one syringe, an equal volume of activated hyaluronate solution (7 mg/ml) containing thrombin (1-5 U/ml) was placed in anther syringe. Both of the syringes were mounted onto a FibriJel™ surgical sealant delivery system connected with a 18 G needle. Gel-matrix containing GDF-5 was formed in less than three minutes at the exit site of the needle upon pushing the two parts of composition to simultaneously flow through the syringes.

The above procedure was followed to make a series of injectable gel-matrices containing other growth factors, hormone, and cytokines.

Example 9

Comparative Sustained Release of GDF-5 from Matrices

Implantable mCOL/HA matrices (5 x 4 x 2 mm) with pre-loaded GDF-5 at the ratio of 50 μg/piece were prepared as described in Example 7, using radio-lebeled GDF-5 as a tracer. Glutaraldehyde cross-linked mCOL and hyaluronate polyaldehyde cross-linked COL (COL/HA) were also loaded with GDF-5 at the same ratio and served as controls. The release kinetics of GDF-5 from these matrices was investigated. One piece of total five pieces of each type of matrix was placed into a 2.0 ml polypropylene tubes containing 5.0 ml PBS, pH 4.0 adjusted by 30 mM acetic acid. The tubes were shaked gently at 37°C. The medium was refreshed at a designated time and the radioactivity in each replaced medium was detected by scintillation counter. As shown in the Figure, the release of GDF-5 from both mCOL/HA and glutaraldehyde linked matrices showed a longer duration than that from COL/HA matrix. Since both mCOL/HA and glutaraldehyde-linked matrices contained 90% of mCOL, while the COL/HA matrix contained the same amount of non-mineralized COL, the results indicated that GDF-5 was bound to mCOL fiber more effectively than bound to COL. Consequently, mCOL/HA matrix is considered superior to COL/HA matrix for GDF-5 sustained delivery.

The above procedure was followed to determine the kinetics of a series of growth factors, hormone, and cytokines released from mCOL/HA matrices.

Example 10

Osteoconductivity of Implanted Matrices

Matrices comprising 9 parts of mCOL and 1 part of HA polyaldehyde (5% repeat units oxidized) were prepared as described in Example 2. Specimens of the matrix with the size of 5 mm x 3 mm x 2 mm were sterilized with ethanol and implanted into the defects created in parietal bone of 6 week old male Spregue-Dawley rats. The defects were evaluated at 28 days by radiographic and histologic analysis, and the results summarized in Table 1. Defects without implantation of matrix showed only a little radiographic reduction (20 ± 2%), defects filled with glutaraldehyde cross-linked mCOL showed a higher reduction (55 ± 3%), defects filled with mCOL/HA showed a further increase in radiopacity (88 ± 3%), which was similar to those of filled with COL/HA matrix (93 ± 4%). The histolofic evaluation correlated with radiographic results. Defects filled with either mCOL/HA or COL/HA showed a higher bone formation score than those filled with mCOL (Table 1). Since both the mCOL/HA and COL/HA matrices contain 10% of HA, which made them different from mCOL prepared from 100%

mCOL, these results demonstrated that the introduction of 10% HA to collagen-based matrices enhanced their osteoconductivity.

Table 1 Radiographic and histological scores of rat cranial defects treated with mCOL/HA, mCOL, and COL/HA matrices

Implant	% Radiographic Reduction in defect Size (mean ± SD)	Histological bone score* (mean ± SD)
Untreated	20 ± 2	1.2 ± 0.5
mCOL/HA	88 ± 3	5
mCOL	55 ± 3	2.7 ± 0.5
COL/HA	93 ± 4	5

***Healing of the defects was scored in the scale of 1-5, based on the width of the defect bridged with reparative bone: 1 = 0 to <20%; 2 = >20 <40%; 3 = >40 <60%; 4 = >60 <80%; 5 = >80%.**

Example 11

In Vitro Growth of Cells and Expression of Bone Phenotype

This example illustrates that the mCOL/HA matrix supports the growth of fetal rat calvarial cells (FRCs) and demonstrates the expression of bone phenotype in vitro. FRCs were prepared from a 19 day old fetus and expanded, seeded into the matrix made by the method described in Example 2 comprising 90% mCOL and 10% HA (5% repeat units were oxidized) and cultured under standard conditions for 4 weeks. Cultures were then evaluated for cell growth and the express of alkaline phosphatase activity (ALP). Results showed that RFCs seeded on the matrix grew continually, and the cell number was increased by 9 fold at day 28, compared to day 1. The expression of ALP, a marker for bone formation, also increased with time and reached the highest value at day 21, indicating the utility for bone formation of the mCOL/HA matrix to guide the seed FRC differentiation.

What is claimed is:

1. A method for preparing a matrix to support the repair of tissue comprising the steps of oxidizing an exogenous polysaccharide to form a modified exogenous polysaccharide having aldehyde groups, and reacting said modified exogenous polysaccharide with mineralized collagen under conditions whereby said aldehyde groups covalently react to crosslink with mineralized collagen to form said matrix.

2. The method of Claim 1 further comprising adding a growth factor to said matrix.

3. The method of Claim 2 wherein said growth factor is selected from the group consisting of members of the TGF-ß superfamily; members of the BMP family; the growth differentiation factors(GDF's); ADMP-1; members of the fibroblast growth factor family; members of the hedgehog family of proteins; members of the insulin- like growth factor (IGF) family; members of the platelet-derived growth factor (PDGF) family; members of the interleukin (IL) family; and members of the colony-stimulating factor (CSF) family.

4. The method of Claim 3 wherein the growth factor is a bone morphogenetic protein (BMP).

5. The method of Claim 1 wherein the polysaccharide comprises hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan, heparan sulfate, dextran, dextran sulfate, or alginate.

6. The method according to Claim 5, wherein said polysaccharide comprises hyaluronic acid.

7. The method according to Claim 1 wherein the collagen is selected from the group consisting of Type 1 and Type II collagen.

8. The method according to Claim 1, wherein said step of oxidizing said polysaccharide comprises treatment of said polysaccharide with periodate.

9. The method according to Claim 1, wherein said mineralized collagen and said polysaccharide used to form said matrix are present in the range of 99:1 to 1:99 by weight, respectively.

10. The method according to Claim 9 wherein said range is 9:1 to 1:9 by weight, respectively.

11. The method according to Claim 1, wherein about 1% to 50% of the repeat units in said polysaccharide are oxidized to contain aldehyde groups.

12. The method according to Claim 11, wherein about 1% to 5% of the repeat units in said polysaccharide are oxidized to contain aldehyde groups.

13. The method according to Claim 1, wherein said matrix is formed by freezing and lyophilization.

14. The method according to Claim 1, wherein said matrix is formed by wet laying and air drying.

15. The method of Claim 1 further comprising adding fibrinogen and thrombin to form fibrin in said matrix.

16. The method of Claim 1 wherein tissue is selected from the group consisting of bone, cartilage and soft tissue.

17. A matrix to support the repair of tissue, said matrix comprising mineralized collagen covalently crosslinked to an exogenous polysaccharide, wherein said polysaccharide is crosslinked to said collagen through oxidized sugar rings on said polysaccharide which form covalent linkages to said mineralized collagen.

18. The matrix of Claim 15 further comprising a growth factor.

19. The matrix of Claim 18 wherein said growth factor is selected from the group consisting of. members of the TGF-ß superfamily; members of the bone morphogenic protein family; the growth differentiation factors(GDF's); ADMP-1; members of the fibroblast growth factor family; members of the hedgehog family of proteins; members of the insulin-like growth factor (IGF) family; members of the platelet-derived growth factor (PDGF) family; members of the interleukin (IL) family; and members of the colony-stimulating factor (CSF) family.

20. The matrix of Claim 18 wherein said growth factor is a bone morphogenetic protein.

21. The matrix according to Claim 17 wherein said polysaccharide comprises hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan, heparan sulfate, dextran, dextran sulfate or alginate.

22. The matrix according to Claim 21 wherein said polysaccharide is hyaluronic acid.

23. The matrix according to claim 17 wherein said matrix comprises said mineralized collagen and said polysaccharide in a weight ratio in the range of 99:1 to 1:99.

24. The matrix of Claim 17 wherein the collagen is selected from the group consisting of Type 1 collagen and Type 2 collagen.

25. The matrix of Claim 17 further comprising fibrin.

26. A method of conducting the growth of bone or cartilage tissue *in vivo* comprising the step of administering a matrix according to claim 17 at a site of desired bone or cartilage growth.

27. A method of inducing the growth of bone or cartilage tissue *in vivo* comprising the step of administering a matrix according to claim 18 at a site of desired bone or cartilage growth.

28. A method of conducting the growth of bone or cartilage tissue *in vivo* comprising the step of administering at the site of desired bone or cartilage growth:

a. an exogenous polysaccharide modified to have aldehyde groups;

b. mineralized collagen; and

c. optionally, a growth factor;

in amounts sufficient to form a matrix at said site to support the growth of bone or cartilage.

29. A method of inducing the growth of bone or cartilage tissue *in vivo* comprising the step of administering at the site of desired bone or cartilage growth:

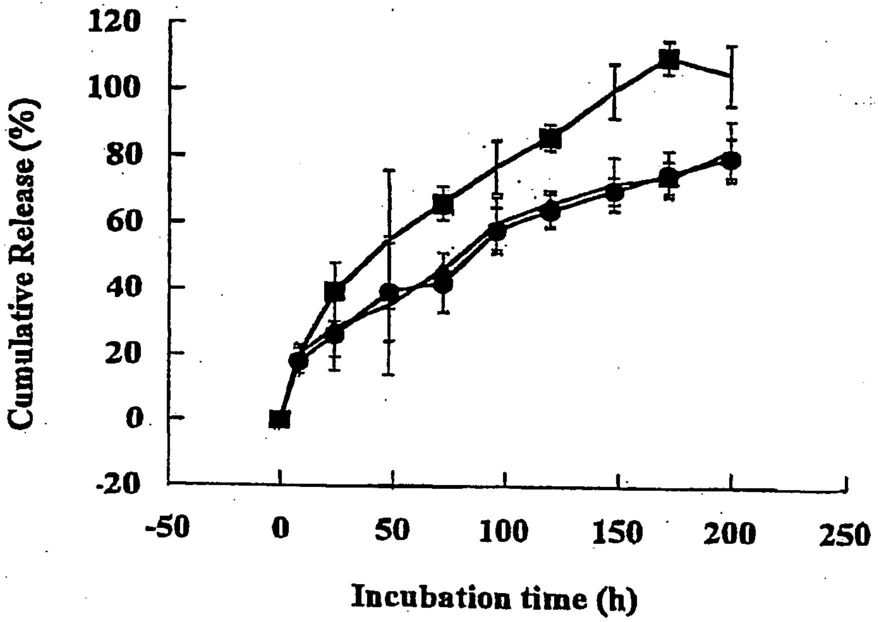
a. an exogenous polysaccharide modified to have aldehyde groups;

b. mineralized collagen; and

c. a growth factor,

in amounts sufficient to form a matrix at said site to support the growth of bone or cartilage.

FIGURE



Cumulative Release of GDF-5 from mCOL/HA (•), mCOL (▲), and COL/HA (■) into PBS of pH 4.0 at 37 °C.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/42477

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 38/16, 38/17, 9/14, 35/14
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/8, 21, 773, 777, 801; 530/356, 382, 395, 813; 424/484, 486, 488

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98/31345 A1 (ORQUEST, INC.) 23 July 1998, abstract, summary of the invention and claims 1-27.	1-29
Y	US 5,866,165 A (LIU et al) 02 February 1999, abstract, summary of the invention and claims 1-20.	1, 5-9, 11-14, 17 and 21-27
Y	US 5,972,385 A (LIU et al) 26 October 1999, abstract, summary of the invention and claims 1-24.	1-17 and 19-26
Y	US 5,231,169 A (CONSTANTZ et al) 27 July 1993, abstract and summary of the invention.	1-29
A,T,E	US 6,309,670 B1 (HEIDARAN et al) 30 October 2001, cols. 3-6.	1-29

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search 11 DECEMBER 2001	Date of mailing of the international search report 22 JAN 2002
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/42477

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/8, 21, 773, 777, 801; 530/356, 382, 395, 813; 424/484, 486, 488

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, DIALOG, MEDLINE, EMBASE, DERWENT, WPIDS
search terms: cartilage? connective tissue? bone?; polysacchardie? aldehyde? periodate?; matric? or matrix? or freez? or lyophil? or wet lay? or air dry?; type I collagen or type II collagen or collagen; hyaluronic acid or chonroitin sulfate or dermatin sulfate or keratin sulfate or heparin or heparin sulfate or alginate; tumor growth factor or TGF or bone morphogenic protein or BMP or growth differentiation factor or GDF or insulin-like growth factor or IGF or platelet derived growth factor or PDGF or interleukin or IL.

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